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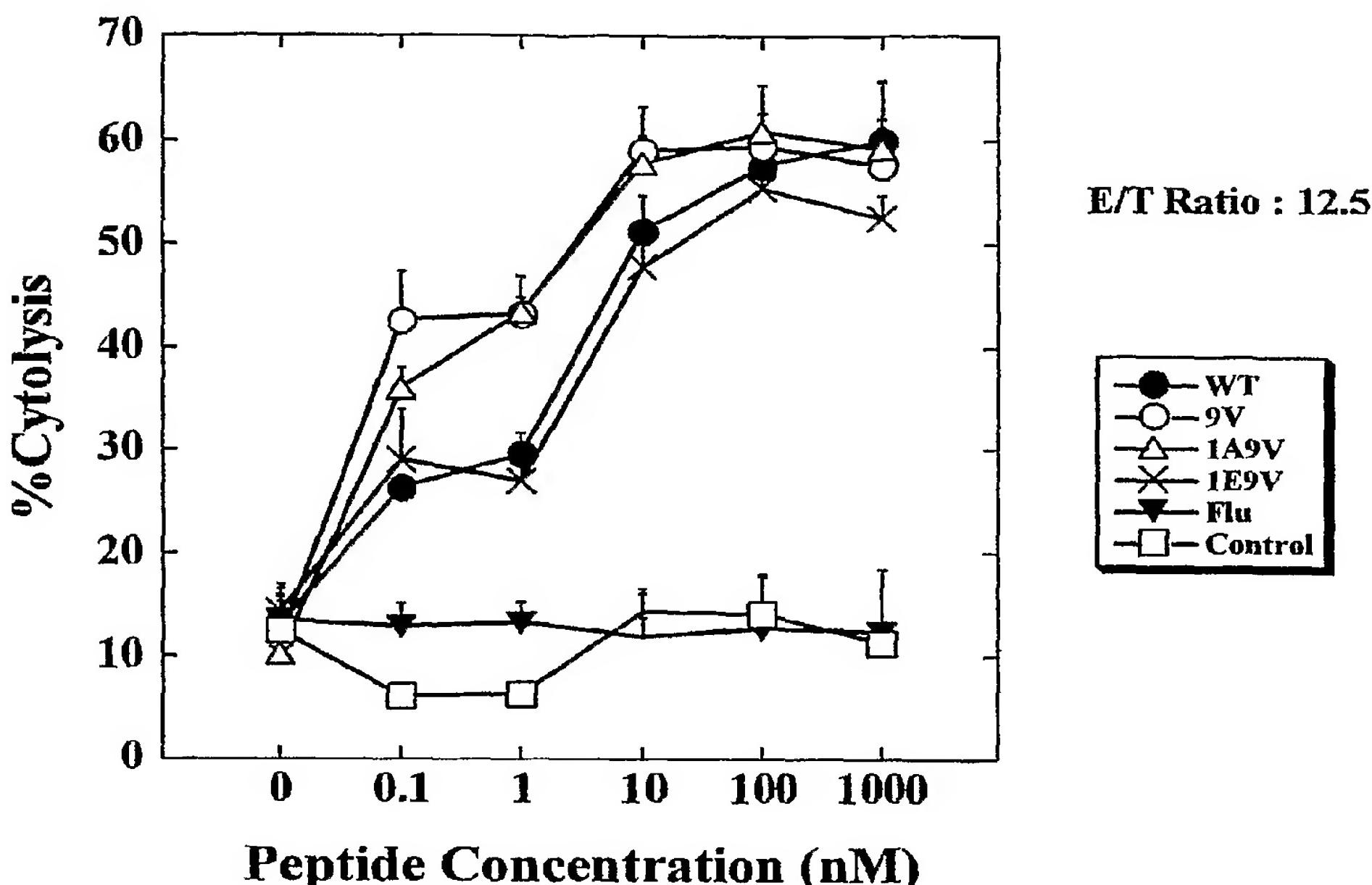
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(54) Title: PEPTIDE ANALOGS CAPABLE OF ENHANCING STIMULATION OF A GLIOMA-SPECIFIC CTL RESPONSE



(57) Abstract: The invention provides a peptide derived from the interleukin-13 receptor $\alpha 2$, which serves as a HLA-A2-restricted cytotoxic T lymphocyte (CTL) epitope. The invention can be used as a vaccine for glioma and can be formulated into compositions for medical or veterinary use. In addition, the invention provides the use of a peptide derived from the Eph family of tyrosine kinase receptors which can be also used as a vaccine for glioma and can be formulated into compositions for medical or veterinary use.

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PEPTIDE ANALOGS CAPABLE OF ENHANCING STIMULATION OF A GLIOMA-SPECIFIC CTL RESPONSE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 60/611,797 filed September 21, 2004, the disclosure of which is incorporated herein in its entirety.

FIELD OF INVENTION

[0002] The invention pertains to reagents and methods for treatment of glioma.

BACKGROUND OF THE INVENTION

[0003] Brain tumors are particularly difficult to treat using conventional methods such as surgery, radiotherapy, or chemotherapy. Factors such as invasive growth patterns and the blood-brain barrier make the treatment of malignant gliomas more problematic than other tumors. The lack of effective treatment options for patients has led to the development of alternative therapies, such as immunotherapy.

[0004] Immunotherapy is a promising new approach in the treatment of malignant gliomas. The efficacy of peripheral immunizations with autologous glioma cells or dendritic cells (DC) pulsed with synthetic peptides for tumor-antigen-specific T cell epitopes has been demonstrated in preclinical mouse models (Okada et al., 2001; Okada et al., 1998). Specific T cell epitope-based vaccines are likely safer than whole glioma cell-based vaccines due to the lack of theoretical autoimmune responses against normal brain components. Such antigen-specific approaches may also be more effective than the bulk tumor-antigen approaches because presentation of immunogenic T cell-epitopes and stimulation of antigen-specific T cell precursors can take place more efficiently with the use of specific antigen-peptides than bulk tumor antigens.

[0005] The identification of T cell immuno-epitopes in human glioma associated antigens is required for the development of such vaccines against human gliomas. Few cytotoxic T lymphocyte (CTL) immuno-epitopes have been identified for human malignant gliomas. However, an HLA (human leukocyte antigen)-A2-restricted cytotoxic T lymphocyte (CTL) epitope derived from the interleukin (IL)-13 receptor (R) α 2 was recently identified (Okano et al., 2002). IL-13R α 2 is known to be expressed in the majority of human malignant

gliomas but not in normal tissues (Debinski et al., 2000), thus making the identified epitope (IL-13R α 2 345-353) an attractive component of peptide-based vaccine for gliomas. By generating unique CTL lines by stimulation of CD8+ cells with the peptide IL-13R α 2 345-353, it was demonstrated that IL-13R α 2 positive, HLA-A2 positive glioma cells were efficiently lysed in an antigen-specific manner. However, it remains unclear how efficiently such peptide-based vaccines can induce specific CTLs and whether peptide-analogues can be used for optimal expansion and activation of IL-13R α 2 specific HLA-A2-restricted CTL.

[0006] It has been demonstrated that certain amino acid substitutions in peptides identified as CTL epitopes could greatly enhance the binding affinity of such peptides to the HLA (human leukocyte antigen) complex and thus would thus augment the immunogenicity of the peptide (Bownds et al., 2001; Chen et al., 2000). The enhancement of the immunogenicity of IL-13R α 2 345-353, and other such epitopes could lead to the development of powerful, tumor-specific peptide-based vaccines, which would be a significant improvement in the current treatment regime for malignant gliomas. However, there remains a need for an improved polypeptide HLA-A2-restricted cytotoxic T lymphocyte (CTL) epitope.

[0007] As discussed above, few cytotoxic T lymphocyte (CTL) immuno-epitopes have been identified for human malignant gliomas. Given the marked antigenic heterogeneity of gliomas, however, immunotherapy with a single tumor-specific T-cell epitope might merely promote transient stabilization of disease, prior to the progression of antigen loss variants. EphA2 is a member of the Eph family of receptor tyrosine kinases, comprised of two major classes (EphA and EphB), which are distinguished by their specificities for ligands (ephrin- A and ephrin-B, respectively). EphA2 is frequently overexpressed and often functionally dysregulated in advanced cancers, such as metastatic lesions (Kinch et al., 2003). Due to the aggressive and invasive nature of malignant gliomas, EphA2 might be expressed in this tumor entity and could be a potential target for glioma vaccines. T-cell immunoepitopes in EphA2 have been identified and characterized as potential targets and surrogate markers for other forms of cancer immunotherapy (Alves et al., 2003, and Tatsumi et al., 2003, the discloses of which are incorporated herein). The identification of additional CTL epitopes is a necessary step in the development of multiepitope-based vaccines for glioma which would be a significant improvement in the current treatment regime for malignant gliomas.

BRIEF SUMMARY OF THE INVENTION

[0008] The invention provides a peptide derived from IL-13R α 2, which serves as a HLA-A2-restricted cytotoxic T lymphocyte (CTL) epitope. The inventive peptide can comprise, consist of, or consist essentially of a substitution mutant variant of WLPFGFILI (SEQ ID NO:1), where least one of the amino acid residues can be substituted for an amino acid other than the indicated residue. In addition, the inventive peptide can comprise, consist of, or consist essentially of any of the following sequences: WLPFGFILV (SEQ ID NO:2), ALPFGFILV (SEQ ID NO:3), or ELPFGFILV (SEQ ID NO:4). The invention also provides a use of any of the above peptides as a vaccine for glioma. In addition, the invention provides a method of vaccinating a patient against glioma, where the peptide is introduced into a patient under conditions sufficient for the patient to develop a CTL response. Further, the invention provides a use of a peptide having the sequence TLADFDPRFV (SEQ ID NO:6) or a composition comprising said peptide and a physiologically acceptable carrier, as a vaccine for glioma. The invention also provides a method of vaccinating a patient against glioma, where a peptide having the sequence TLADFDPRFV (SEQ ID NO:6) or a composition comprising said peptide and a physiologically acceptable carrier, is introduced into a patient under conditions sufficient for the patient to develop a CTL response. These and other advantages of the invention, as well as additional inventive features, will be apparent from the accompanying drawings and the description of the invention provided herein.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0009] Figure 1 graphically presents data demonstrating that IL-13R α 2-V9 and IL-13R α 2-A1V9 induced a higher magnitude of CTL reactivity than the native IL-13R α 2₃₄₅₋₃₅₃ or IL-13R α 2-E1V9 against T2 cells loaded with various concentrations of native IL-13R α 2₃₄₅₋₃₅₃. CD8+ T cells from an HLA-A2+ glioma patient were stimulated with DCs loaded with either, native IL-13R α 2₃₄₅₋₃₅₃ (●), IL-13R α 2-V9 (○), IL-13R α 2-A1V9 (Δ), IL-13R α 2-E1V9 (X), Influenza M1₅₈₋₆₆ peptide (▼), or no peptide (□) for 10 days. Then, the T cells were tested for lytic activity against T2 cells loaded with indicated concentrations of IL-13R α 2₃₄₅₋₃₅₃ or no peptide by 4-hr ⁵¹Cr-release assay. The E/T ratio was 12.5. P<0.01 for IL-13R α 2-V9 vs. native as well as IL-13R α 2-A1V9 vs. native at 0.1 and 1 nM by two-tailed Student-t test. These data demonstrate results from one of three separate experiments with similar results.

[0010] Figure 2 graphically presents data demonstrating that the CTL line induced by the V9 peptide had increased lytic activity against T2 cells loaded with various concentrations of the wild type IL-13R α 2₃₄₅₋₃₅₃ peptide. The CTL lines induced by each of the 3 agonist analogues or the wild type peptide were examined for CTL activities against lower concentrations of target IL-13R α 2₃₄₅₋₃₅₄ peptide with T2 cells loaded with various concentrations (1 – 100 nM) of IL-13R α 2₃₄₅₋₃₅₄ by 4-Hr ⁵¹Cr-release assay (E/T ratio = 50).

[0011] Figure 3 graphically presents data demonstrating that the modified peptides induced a higher magnitude of CTL reactivity than the native IL-13R α 2₃₄₅₋₃₅₃ against human glioma cell lines. CD8+ cells derived from an HLA-A2+ glioma patient were stimulated with native IL-13R α 2₃₄₅₋₃₅₃ (●), IL-13R α 2-V9 (○), IL-13R α 2-A1V9 (Δ), or IL-13R α 2-E1V9 (X). On day 10, the cells were tested for lytic ability against human glioma cells SNB19 and U-251 (both are IL-13R α +/HLA-A2+) using 4-Hr ⁵¹Cr-release assay. Against SNB19 glioma cells, p<0.05 at all E/T ratios for IL-13R α 2-V9 vs. native IL-13R α 2₃₄₅₋₃₅₃ as well as IL-13R α 2-A1V9 vs. native IL-13R α 2₃₄₅₋₃₅₃ by two-tailed Student-t tests. Against U251 glioma cells, p<0.05 at E/T ratio of 10 and 40 for IL-13R α 2-V9 vs. native IL-13R α 2₃₄₅₋₃₅₃ as well as IL-13R α 2-A1V9 vs. native IL-13R α 2₃₄₅₋₃₅₃ by two-tailed Student-t tests. IL-13R α 2-E1V9 did not improve the CTL reactivity for a statistically significant level in comparison to the native. The data presented represent one of three experiments with different donors with similar results.

[0012] Figure 4 graphically presents data demonstrating that the addition of "cold" T2 cells pulsed with IL-13R α 2₃₄₅₋₃₅₃ inhibited the CTL activities indicating the antigen-specificity of the CTL lines. The CTL lines induced with each peptide were incubated for 4 h with ⁵¹Cr -labeled human glioma cell lines SNB19 at the indicated E:T ratios for evaluation of specific lytic ability (●). For the cold target inhibition assay, ⁵¹Cr -labeled target SNB19 cells (1×10^3 cells/well) and cold T2 cells (1×10^4 cells/well) pulsed with (Δ) or without (○) peptide IL-13R α 2₃₄₅₋₃₅₃ were incubated with the CTLs.

[0013] Figure 5 graphically presents data demonstrating that the addition of anti-HLA-A2 antibody inhibited the CTL activities indicating HLA-A2-restricted recognition of the CTL lines. The CTL lines induced with each peptide were incubated for 4 h with ⁵¹Cr -labeled human glioma cell line SNB19 at the indicated E:T ratios for evaluation of specific lytic ability (●).

Anti-HLA-A2 antibody (W6/32; 10 µg/ml) was added to block the function of HLA-A2 mediated recognition by the T cells (○).

[0014] Figure 6 graphically presents data demonstrating that the modified peptides induced higher magnitude of CTL reactivity than the native IL-13R α 2₃₄₅₋₃₅₃ against EL4-HHD loaded with the native IL-13R α 2₃₄₅₋₃₅₃. SPCs obtained from HHD mice that had been immunized with either control MART-1₂₇₋₃₅ (●), native IL-13R α 2₃₄₅₋₃₅₃ (○), IL-13R α 2-V9 (Δ) or IL-13R α 2-AIV9 (X) were tested for their specific lytic activity against EL4-HHD cells pulsed with the native IL-13R α 2₃₄₅₋₃₅₃ by standard 4 hr-⁵¹Cr-release assays.

[0015] Figure 7 graphically presents data demonstrating that the modified peptides induced a higher magnitude of CTL reactivity than the native IL-13R α 2₃₄₅₋₃₅₃ against EL4-HHD- IL-13R α 2. SPCs obtained from HHD mice that had been immunized with either control MART-1₂₇₋₃₅ (A), native IL-13R α 2₃₄₅₋₃₅₃ (B), IL-13R α 2-V9 (C), or IL-13R α 2-AIV9 (D) were tested their specific lytic activity against EL4-HHD- IL-13R α 2 (○) or control EL4-HHD (●) by standard 4 hr-⁵¹Cr-release assays.

[0016] Figure 8 depicts the expression of EphA2 protein in glioblastoma multiforme (GBM) and anaplastic astrocytoma (AA). Paraffin embedded sections of surgical specimens obtained from patients with GBM (A-C) or AA (D) were deparaffinized and stained with anti-EphA2 polyclonal antibody (C-20: Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or control rabbit IgG (upper right corner window for each sample). Relatively dense staining on endothelia and tumor cells surrounding the vessel was observed (D). Nine of fourteen GBM and six of nine AA cases examined were positive for EphA2 (not shown). Original magnification; x 20.

[0017] Figure 9 graphically presents data demonstrating that the CD8+ cells stimulated with EphA2₈₈₃₋₈₉₁ elicited CTL responses against human glioma cells expressing HLA-A2 and EphA2 protein. CD8+ T cells from an HLA-A2+ glioma patients were stimulated with DCs loaded with EphA2₈₈₃₋₈₉₁ for 10 days. These T cells were then tested for their lytic activity against human glioma cells SNB19 (HLA-A2+, EphA2+), U251 (HLA-A2+, EphA2+) and A172 (HLA-A2-, EphA2+). by 4-hr ⁵¹Cr-release assay.

DETAILED DESCRIPTION OF THE INVENTION

[0018] In one embodiment, the invention provides an isolated peptide that comprises, consists of, or consists essentially of an amino acid sequence comprising, consisting of, or

consisting essentially of a substitution mutant variant of WLPFGFILI (SEQ ID NO:1), wherein at least one of the amino acid residues in SEQ ID NO:1 is substituted with an amino acid other than the indicated residue. For example, at least two (e.g., three or more, four or more, etc.) of the amino acid residues in SEQ ID NO:1 can be substituted with an amino acid other than the indicated residue. Preferably, however, the inventive polypeptide contains a substitution of only one or two of the amino acids. Moreover, while the inventive polypeptide can comprise such a sequence of amino acid, more preferably, the inventive polypeptide consists essentially of such a sequence, and even more preferably, the inventive polypeptide consists of such sequence (i.e., such that the inventive polypeptide consists of nine amino acids).

[0019] The substitution from SEQ ID NO:1 can be, but need not be, a conservative substitution. Conservative substitutions are well known in the art and can be amino acid replacements that preserve the structure and functional properties of proteins, such as the substitution of one or more amino acids by similar amino acids. For example, a conservative substitution can be the substitution of an amino acid for another amino acid within the same general class (e.g., an acidic amino acid, a basic amino acid, or a neutral amino acid).

[0020] Preferred embodiments of the inventive peptide include substituting the W in the first position of SEQ ID NO:1 with an amino acid other than W, substituting the I in the ninth position of SEQ ID NO:1 with an amino acid other than I, or any combination thereof. For example, the W in the first position of SEQ ID NO:1 can be substituted with either A or E, and/or the I in the ninth position of SEQ ID NO:1 can be substituted with V. Preferred examples of the inventive peptide include isolated peptides comprising, consisting of, or consisting essentially of a sequence of amino acid residues comprising, consisting of, or consisting essentially of WLPFGFILV (SEQ ID NO:2), ALPFGFILV (SEQ ID NO:3), or ELPFGFILV (SEQ ID NO:4).

[0021] The inventive peptide can be prepared by methods known to those of ordinary skill in the art. For example, the inventive peptide can be synthesized using solid phase peptide synthesis techniques (e.g., Fmoc). Alternatively, the peptide can be synthesized using recombinant DNA technology (e.g., using bacterial or eukaryotic expression systems). Accordingly, to facilitate such methods, the invention provides genetic vectors (e.g., plasmids) comprising a sequence encoding the inventive peptide, as well as host cells comprising such vectors. Methods for solid state protein synthesis and recombinant protein synthesis are well-known in the art. For example, "Molecular Cloning, A Laboratory

Manual" (Sambrook et al., 3d Ecition, Cold Spring Harmor Press), is a well-known reference detailing many suitable techniques for recombinant production of polypeptides. Accordingly, the invention provides the inventive peptide in recombinant form.

[0022] However it is made, the inventive peptide can be isolated and/or purified (or substantially isolated and/or substantially purified). Accordingly, the invention provides the inventive peptide in substantially isolated form (i.e., substantially isolated from other polypeptides or impurities). The peptide can be isolated from other peptides as a result of solid phase protein synthesis, for example. Alternatively, the peptide can be substantially isolated from other proteins after cell lysis from recombinant production. Standard methods of protein purification (e.g., HPLC) can be employed to substantially purify the inventive peptides. Thus, a preparation of the inventive polypeptide preferably is at least 90% free of other polypeptides and/or contaminants, and more preferably is at least about 95% free of other polypeptides and/or contaminants (such as at least about 97% or 98% free of other polypeptides and/or contaminants). In a most preferred embodiment, the invention provides a preparation of the inventive polypeptide that is greater than 99% free of other polypeptides and/or contaminants (e.g., greater than 99.5% or even 99.9% or even 99.99% free of other polypeptides).

[0023] In another embodiment, the invention provides a preparation of the inventive peptide in a number of formulations, depending on the desired use. For example, where the peptide is substantially isolated (or even nearly completely isolated from other proteins), it can be formulated in a suitable medium solution for storage (e.g., under refrigerated conditions or under frozen conditions). Such preparations can contain protective agents, such as buffers, preservatives, cryoprotectants (e.g., sugars such as trehalose), etc. The form of such preparations can be solutions, gels, etc., and the inventive peptide can, in some embodiments, be prepared in lyophilized form. Moreover, such preparations can include other desired agents, such as small molecules or even other peptides and proteins, if desired. Indeed, the invention provides such a preparation comprising a mixture of different embodiments of the inventive peptide (e.g., a plurality of peptide species as described herein). Technology for preparing such compositions (e.g., lyophilization, preparation of protein solutions, etc.), is within the state of the art.

[0024] In another embodiment, the invention provides a composition comprising, consisting of, or consisting essentially of one or more of the inventive peptides (including mixtures thereof) and a physiologically acceptable carrier or a pharmaceutically acceptable

carrier. Any carrier which can supply the peptide without destroying the vector within the carrier is a suitable carrier, and such carriers are well known in the art. The composition can be introduced to a patient in using any suitable method which allows the patient to develop a CTL response. Such methods are well known in the art and include, for example, parenteral, oral, and topical administration. For example, a parenteral formulation could consist of a prompt or sustained release liquid preparation, dry powder, emulsion, suspension, or any other standard formulation. An oral formulation of the pharmaceutical composition could be, for example, a liquid solution, such as an effective amount of the composition dissolved in diluents (e.g., water, saline, juice, etc.), suspensions in an appropriate liquid, or suitable emulsions. An oral formulation could also be delivered in tablet form, and could include excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients. A topical formulation could include compounds to enhance absorption or penetration of the active ingredient through the skin or other affected areas, such as dimethylsulfoxide and related analogs. The physiological or pharmaceutical composition could also be delivered topically using a transdermal device, such as a patch, which could include the composition in a suitable solvent system with an adhesive system, such as an acrylic emulsion, and a polyester patch.

[0025] In addition to the inventive peptide carrier, as discussed above, the inventive composition can further comprise, consist of, or consist essentially of a T-helper epitope. Any suitable T-helper epitope which stimulates an immune response, such as for example the stimulation of a CD4+ helper T cells and/or CD8+ CTLs, can be used. In a preferred embodiment, the T-helper epitope can be or comprise a peptide comprising, consisting of, or consisting essentially of a sequence of amino acids comprising, consisting of, or consisting essentially of TPPAYRPPNAPIL (SEQ ID NO:5).

[0026] In another embodiment, the invention provides a use of the inventive peptide or composition as a prophylactic or therapeutic vaccine for glioma. The inventive peptide can be used to treat any type of glioma, such as ependymomas, astrocytomas, oligodendrogiomas, glioblastomas, or mixed gliomas, or prophylactically in the prevention of such diseases. Thus, the invention includes the use of the inventive peptide and/or composition for preparation of a medicament useful for vaccinating a patient against glioma.

[0027] The invention further provides a method of vaccinating a patient against glioma, comprising, consisting of, or consisting essentially of introducing into the patient the inventive peptide or composition under conditions sufficient for said patient to develop a

CTL response. As noted above, the composition can include a T-helper epitope, and inclusion of such epitope in the composition for use in the inventive method is preferred, but not necessary.

[0028] In another embodiment, the invention provides a use of a peptide having (e.g., comprising, consisting essentially of, or consisting of) the amino acid sequence TLADFDPRFV (SEQ ID NO:6) as a prophylactic or therapeutic vaccine for glioma. The invention also provides a use of a composition comprising, consisting of, or consisting essentially of a peptide having the amino acid sequence TLADFDPRFV (SEQ ID NO:6) and a physiologically acceptable carrier as such a prophylactic or therapeutic vaccine. Thus, the invention provides the use of such peptide for preparation of a medicament useful for vaccinating a patient against glioma.

[0029] This aspect of the invention further provides a method of vaccinating a patient against glioma, comprising, consisting of, or consisting essentially of introducing into the patient a peptide having (e.g., comprising, consisting essentially of, or consisting of) the amino acid sequence TLADFDPRFV (SEQ ID NO:6) under conditions sufficient for said patient to develop a CTL response. In another embodiment, the invention provides a method of vaccinating a patient against glioma, comprising, consisting of, or consisting essentially of introducing into the patient a composition comprising, consisting of, or consisting essentially of a peptide having the sequence TLADFDPRFV (SEQ ID NO:6) and a physiologically acceptable carrier. The physiological carrier can be a pharmaceutically acceptable carrier. As discussed above, any carrier which can supply the peptide without destroying the vector within the carrier is a suitable carrier, and such carriers are well known in the art. The composition can include a T-helper epitope, as noted above, and inclusion of such epitope in the composition for use in the inventive method is preferred, but not necessary. The peptide can be used to treat any type of glioma, such as ependymomas, astrocytomas, oligodendrogiomas, glioblastomas, or mixed gliomas, or prophylactically in the prevention of such diseases.

[0030] In accordance with the inventive method, regardless of the exact peptide used or the formulation of the composition, the patient can be any individual that has been diagnosed with glioma or is identified as at risk for developing glioma. In a preferred embodiment, the patient is a mammal. Even more preferably, the patient is human.

[0031] In accordance with the inventive method, the peptide or composition can be introduced to the patient by any suitable method, such as those described above. For

therapeutic use, the peptide preferably is introduced locally into the situs of the glioma or systemically in amounts sufficient to treat the glioma. For prophylactic use, the peptide or composition is introduced into a patient in any suitable manner to deliver a sufficient amount of the protein to the patient to achieve a protective effect.

[0032] For therapeutic use, following introduction of the peptide or composition into the patient, in accordance with the inventive method, the patient's condition is monitored to assess the severity of the glioma. Suitable application of the inventive method will result in slowing of the progression of the glioma and, in preferred embodiments, result in plateauing of the progress of the disease. Indeed, in more preferred embodiments, application of the inventive method will result in shrinkage of glioma in the patient or even in substantial or complete remission of the glioma. Thus, while the inventive method can lead to a cure of the glioma in some patients, any degree of improvement in the prognosis of the patient following application of the inventive method is considered to be successful application. Moreover, it is to be understood that the inventive method can be used as monotherapy or adjunctively in combination with other therapeutic agents (e.g., chemotherapy or radiotherapy) or therapeutic methods.

[0033] For prophylaxis, following introduction of the inventive polypeptide or composition into the patient, the patient is suitably monitored to assess the development of glioma and/or continued risk of developing glioma. Successful prophylaxis can be measured by the absence of glioma in the patient for longer than the initial assessment of risk had predicted.

EXAMPLES

[0034] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope. Briefly, the examples demonstrate the generation of a potent agonist analogue peptide identified here, which can induce IL-13R α 2-specific CTL responses even more efficiently than the original IL-13R α 2 345-353. Three IL-13R α 2 345-353 analogue peptides have been created by substituting the carboxy-terminal isoleucine (I) for valine (V) and the amino terminal tryptophan (W) for either alanine (A), glutamic acid (E), or non-substituted (W) (designated as A1V9, E1V9 and V9, respectively). Relative immunogenicity of these IL-13R α 2 (345-353) peptide analogues was examined by stimulating peripheral blood cells (PBL) from glioma patients with dendritic cells (DC) loaded with each of these peptides. Among the peptide analogues tested, V9 was capable of inducing IL-13R α 2 345-353 specific CTL from PBL of glioma patients more efficiently than

IL-13R α 2₃₄₅₋₃₅₃ consistently in all donors. These findings suggest that a highly antigenic IL-13R α 2 peptide-analogue V9 will be useful for the development of vaccines capable of expanding IL-13R α 2 specific CTL in glioma patients. The following examples also demonstrate the ability of the peptide EphA2₈₈₃₋₈₉₁ to induce an EphA2 specific CTL response, thus suggesting that the peptide would be useful in the development of additional vaccines for glioma.

EXAMPLE 1

[0035] This example demonstrates the identification of modified peptides for IL-13R α 2₃₄₅₋₃₅₃ that enhance induction of the CTL response against native IL-13R α 2₃₄₅₋₃₅₃.

[0036] Three modified peptides were synthesized as listed in Table 1. The binding capability of these modified peptides was assessed using an HLA-A2 transfected T2 cell line. Aliquots of T2 cells were incubated with modified peptides or IL-13R α 2₃₄₅₋₃₅₃ at 1 nM overnight, and then examined for the surface expression levels of HLA-A2 on T2 cells by flow cytometry. Since stable binding of HLA-A2 with peptide epitopes further stabilizes the surface expression of HLA-A2 (Francini et al., 2002; Alves et al., 2003), quantitative expression levels of HLA-A2, which is indicated by Mean Fluorescence Intensity (MFI) in Table 1, correlate with the binding affinity of the peptide-epitopes that are co-incubated with the T2 cells. The modified peptides V9 and A1V9 possess higher binging affinity to HLA-A2 than the native IL-13R α 2₃₄₅₋₃₅₃ (Table 1), suggesting the possibility that these modified peptides are more immunogenic than the IL-13R α 2₃₄₅₋₃₅₃.

Peptide	Amino Acid Sequence	Binding Index(MFI*)	Designation
WLPFGFILI (SEQ ID NO:1)	native IL-13R α 2 ₃₄₅₋₃₅₃	237.4	native
WLPFGFILV (SEQ ID NO:2)	V9: I was replaced with V at P9	375.6	V9
ALPFGFILV (SEQ ID NO:3)	A1V9: W→A at P1, and I→V at P9	462.8	A1V9
ELPFGFILV (SEQ ID NO:4)	E1V9: W→E at P1, and I→V at P9	241.6	E1V9
(Control : Non peptide)		121.8	

* Mean Fluorescence Intensity at the peptide concentration of 1 nM

EXAMPLE 2

[0037] This example demonstrates that CTL induced by the agonist analogue V9 recognized peptide IL-13R α 2₃₄₅₋₃₅₄ presented on HLA-A*0201 more efficiently than CTL induced by the wild type peptide.

[0038] Dendritic cells (DCs) derived from HLA-A*0201⁺ glioma patients were pulsed with either V9, A1V9, E1V9, a control influenza (flu), or the wild type peptide (10 µg/ml), and used to stimulate autologous CD8⁺ T cells. On day 7, the individual responder cell cultures were then restimulated once with autologous DCs loaded with the corresponding peptide used in the primary stimulation. Specific CTL activity of the induced T cell lines was first tested with T2 cells loaded with the wild type IL-13R α 2₃₄₅₋₃₅₃, or no peptide on day 10.

[0039] As depicted in Figure 1, the T cells that had been stimulated with either wild type (IL-13R) or agonist analogues (V9, A1V9 and E1V9) efficiently lysed T2 target cells pulsed with 100 ng/ml wild type IL-13R α 2₃₄₅₋₃₅₃; whereas only low background lysis was observed in the absence of the peptide on T2 cells. T cells that had been stimulated with the control flu-peptide or no-peptide (control) did not demonstrate any lytic activity over background levels. These results demonstrated that the CTL lines induced with the wild type or agonist analogues recognized and lysed the cells presenting wild type IL-13R α 2₃₄₅₋₃₅₃ epitope specifically. In particular, the V9 peptide induced a significantly higher level of antigen-specific CTL response in comparison to the wild type IL-13R α 2₃₄₅₋₃₅₃ at each effector/target (E/T) ratio (p= 0.018, 0.020 and 0.011 at an E/T ratio of 50, 25 and 12.5, respectively). The same set of experiments were repeated with at least three individual HLA-A2+ glioma patients, and the V9 peptide consistently demonstrated higher CTL activities than the native IL-13R α 2₃₄₅₋₃₅₃ in all four donors tested (data not shown).

[0040] Subsequently, the sensitivity of the CTL lines induced by agonist analogues or the wild type peptide was examined with T2 cells loaded with various concentrations (1 –100 nM) of the IL-13R α 2₃₄₅₋₃₅₄ peptide by 4-Hr ⁵¹Cr-release assay (Figure 2). All CTL lines demonstrated peptide-dose dependent lytic activities against peptide-loaded T2 cells. The CTL line induced by the agonist analogue V9 demonstrated higher CTL activities than the wild type IL-13R α 2₃₄₅₋₃₅₄ at all peptide-concentrations examined (P =0.029, 0.039 and 0.018 at 1, 10 and 100 nM, respectively). It is noteworthy that the average percent lysis value achieved by V9-induced CTL with 1 nM IL-13R α 2₃₄₅₋₃₅₄ was higher than that demonstrated with wild type peptide-induced CTL with 100 nM peptide, although this did not demonstrate

a statistical significance due to a large standard variation. These results indicate that the V9 peptide is more efficient than the wild type peptide in inducing CTL that are capable of recognizing low concentrations of the target wild type IL-13R α 2₃₄₅₋₃₅₃ peptide. This ability is important because human tumor cells express low levels of target CTL epitopes on their HLA-molecules (Bakker et al., 1995; Lupetti et al., 1998).

EXAMPLE 3

[0041] This example demonstrates that CTL induced by modified peptides lysed HLA-A2+ glioma cells that express IL-13R α 2 more efficiently than CTL induced by the native peptide.

[0042] The ability of modified peptides, such as IL-13R α 2-V9, to enhance the CTL activity against HLA-A2+ human glioma cells that endogenously expressed and presented IL-13R α 2-derived epitopes was examined. Human glioma cell lines U251 and SNB19 express HLA-A2 and IL-13R α 2, whereas human glioma cell line A172 expresses IL-13R α 2 but not HLA-A2 (Okano et al., 2002). Therefore, U251 and SNB19 were used as relevant target glioma cells, while A172 served as a negative control line to demonstrate HLA-A2-restriction of the response.

[0043] The lytic ability of the peptide-induced CTL lines against these glioma cells was examined using 4-hr ⁵¹Cr-release assays. As illustrated in Figure 4, the U-251 and SNB19 cell lines were highly susceptible to cytotoxic activity of the all CTL lines that had been induced with IL-13R α 2₃₄₅₋₃₅₃ or each of its modified peptides. A172 cells, in contrast, were not lysed beyond the background level (<10%) by any of the CTL lines tested, suggesting that the IL-13R α 2₃₄₅₋₃₅₃ or modified peptide-induced CTL lines lysed SNB19 and U-251 glioma cells in an HLA-A2 restricted manner (data not shown). The T cells stimulated with a melanoma associated antigen epitope Mart-1 27-35 and T cells with no peptide stimulation showed only background level (<10%) lysis at all Effector/Target (E/T) ratios tested (data not shown). In this particular patient, both IL-13R α 2-V9 and -A1V9 induced higher levels of lysis of SNB19 and U-251 in each E/T ratio in comparison to the native IL-13R α 2₃₄₅₋₃₅₃ peptide.

[0044] To determine the specificity of the lytic activity, cold target competition experiments were performed by addition of non-radiolabeled (clod) T2 cells pulsed with IL-13R α 2₃₄₅₋₃₅₃ peptide in the 4-h ⁵¹Cr-release assay (Figure 4). The anti-SNB19 glioma cell lytic activities by the CTL lines induced by the native IL-13R α 2₃₄₅₋₃₅₃ or IL-13R α 2-V9

were almost completely inhibited by the addition of the cold T2 cells pulsed IL-13R α 2 345-353. The CTL activities, however, were not inhibited by the addition of non-peptide pulsed cold T2 cells, demonstrating that the lytic ability of the CTLs was specific for the epitope IL-13R α 2 345-353.

[0045] Furthermore, anti-HLA-A2 antibody (W6/32) was used to block the HLA-A2 mediated signaling in the CTL reactivity. As illustrated in Figure 5, addition of this antibody inhibited the CTL-mediated lysis, confirming that the anti-glioma CTL reactivity induced by these peptides was HLA-A2 restricted.

EXAMPLE 4

[0046] This example demonstrates the vaccination of HLA-A2 transgenic (HHD) mice with IL-13R α 2-derived CTL epitopes.

[0047] In order to examine whether immunization with IL-13R α 2 345-353 and/or its modified peptides can elicit CTL responses *in vivo*, and also to examine whether induced CTL responses can mediate therapeutic anti-tumor responses against IL-13R α 2 345-353-expressing brain tumors, the HHD mice were obtained from Dr. Francois A. Lemonnier (Pasteur Institute, Paris). HHD mice are D^bx β 2 microglobulin (β 2M) null, and transgenic for modified HLA-A2.1- β 2 microglobulin single chain (*HHD* gene) (Pascolo et al., 1997). *In vivo* experiments showed that HHD mice exhibit HLA-A2-restricted responses to multiepitope proteins such as intact influenza virus (Pascolo et al., 1997) and novel cancer associated antigens, such as EphA2 (Alves et al., 2003), HER-2/neu and hTERT (Scardino et al., 2002), MAGE (Graff-Dubois et al., 2002) and a novel breast carcinoma associated BA46 (Carmon et al., 2002). Hence, these mice are a useful tool for the identification and characterization of potential tumor-derived, HLA-A2-restricted CTL epitopes.

[0048] To create an HHD mouse-syngeneic tumor cell line that expresses IL-13R α 2, HHD gene-transfected EL4 lymphoma cells (EL4-HHD) were obtained. EL4-HHD cells have been generated from EL4 by depletion of D^bx62M and insertion of modified HLA-A2.1-B2M single chain (Pascolo et al., 1997), thereby allowing syngeneic transplantation in HHD mice. EL4-HHD cells were stably transfected with an expression plasmid encoding IL-13R α 2. The cell line (EL4-HHD-IL-13R α 2) expressed IL-13R α 2 protein and formed tumors both in subcutaneous (s.c.) and intracranial (i.e.) space following injections to syngeneic HHD mice.

EXAMPLE 5

[0049] This example demonstrates that *in vivo* immunization of HHD mice with the modified peptides induced higher magnitudes of CTL responses than the native peptide against the target cells expressing IL-13R α 2₃₄₅₋₃₅₃.

[0050] HHD mice received (on days 7 and 14) s.c injections of 100 μ g of peptide IL-13R α 2-V9, -A1V9, IL-13R α 2₃₄₅₋₃₅₃, or MART-1₂₇₋₃₅) emulsified in incomplete Freund's adjuvant (IFA) in the presence of 140 μ g of the I-A^b-restricted HBVcore₁₂₈ (TPPAAYRPPNAPIL) (SEQ ID NO:5) T-helper epitope, which stimulates a CD4+ helper T cell response, thereby promoting the stimulation of CD8+ CTLs. Control animals received IFA containing HBV helper-peptide only. Eleven days after the last immunization, the animals were sacrificed, and 5 x 10⁶ spleen cells (SPCs) were stimulated *in vitro* with the same peptide that was used for *in vivo* stimulation (10 jоM). On day 6 of culture, the bulk populations were tested for specific cytotoxicity against the EL4-HHD cells expressing IL-13R α 2 or EL4-HHD pulsed with IL-13R α 2₃₄₅₋₃₅₃.

[0051] EL4-HHD- IL-13R α 2 and EL4-HHD were labeled with 100 μ Ci of ⁵¹Cr for 60 min, plated in 96-well V-bottomed plates (3 x 10³ cell/well). Labeled EL4-HHD were pulsed with IL-13R α 2₃₄₅₋₃₅₃ (1 μ M) at 37°C for 2 h. Control target cells were pulsed with no peptides. Stimulated SPCs were then added as effector cells and incubated at 37°C for 4 h. One hundred μ l of supernatant were collected and radioactivity measured in a gamma counter.

[0052] Figure 6 demonstrates that the CTL responses induced by the modified peptides were able to lyse T2 cells loaded with the native IL-13R α 2₃₄₅₋₃₅₃. Control non-pulsed EL4-HHD cells were not lysed by the CTLs beyond background levels (shown in Figure 7). Furthermore, the immunization with IL-13R α 2-V9 displayed a trend toward higher levels of CTL reactivity against the EL4-HHD cells pulsed with the native IL-13R α 2₃₄₅₋₃₅₃ peptide than other peptides examined, although the difference was not statistically significant due to the variation within the triplicated samples. These data support the previous set of data with human HLA-A2+ patient derived T cells, in which the modified peptides induced higher levels of anti- IL-13R α 2₃₄₅₋₃₅₃ CTL response than the native peptide.

[0053] The ability of the same HHD mice-derived CTLs used in Figure 6 to lyse EL4-HHD-IL-13R α 2 cells was examined in order to evaluate the ability of the CTLs to recognize the IL-13R α 2₃₄₅₋₃₅₃ peptide that is naturally processed by cells that endogenously express IL-13R α 2. Figure 7 illustrates that immunization with the IL-13R α 2₃₄₅₋₃₅₃, IL-13R α 2-V9 or -A1V9

induced a specific CTL activity against EL4-HHD- IL-13R α 2 cells. The CTL activities were antigen-specific because control EL4-HHD were not lysed beyond the background level. Modified peptides IL-13R α 2-V9 and -A1V9 induced higher magnitude of CTL activities in comparison to native IL-13R α 2 345-353 against the EL4-HHD- IL-13R α cells ($p < 0.05$ at all effector/target ratios). The *in vivo* anti-tumor effect of vaccinations with the IL-13R α 2 345-353 or modified IL-13R α 2 peptides in HHD mice bearing EL4-HHD- IL-13R α 2 tumors is currently being evaluated.

EXAMPLE 6

[0054] This example demonstrates that EphA2 has available HLA-A2-restricted CTL epitopes.

[0055] EphA2 is an attractive tumor-associated antigen and a target for tumor-vaccines, as 5 HLA-A2 and 3 DR4 T cell epitopes have been previously identified (Tatsumi et al., 2003). As shown in Figure 8, 9 of 14 human glioblastoma multiforme (GBM) and 6 of 9 anaplastic astrocytoma (AA) cases express high levels of EphA2. In addition, anti-glioma CTL reactivity has been induced in CD8+ cells obtained from HLA-A2+ glioma patients by stimulation with the EphA2 883-891 epitope (Figure 9). This response was specific for the EphA2 883-891 epitope because the parallel assay using T2 cells loaded with EphA2 883-891 demonstrated a peptide-specific response in comparison to the control unloaded T2 target (not shown). These data strongly suggest that EphA2 883-891 can serve as a CTL epitope.

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[0056] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0057] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and

“containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0058] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIM(S):

1. An isolated peptide comprising an amino acid sequence comprising a substitution mutant variant of WLPFGFIL (SEQ ID NO:1), wherein at least one of the amino acid residues in SEQ ID NO:1 is substituted with an amino acid other than the indicated residue.
2. The isolated peptide of claim 1, wherein at least two of the amino acid residue in SEQ ID NO:1 is substituted with an amino acid other than the indicated residue.
3. The isolated peptide of claim 1, wherein the W in the first position of SEQ ID NO:1 is substituted with an amino acid other than W.
4. The isolated peptide of claim 1, wherein the I in the ninth position of SEQ ID NO:1 is substituted with an amino acid other than I.
5. The isolated peptide of claim 1, wherein the substitution is a conservative substitution.
6. The isolated peptide of claim 1, wherein the W in the first position of SEQ ID NO:1 is substituted with either A or E.
7. The isolated peptide of claim 1, wherein the I in the ninth position of SEQ ID NO:1 is substituted with V.
8. An isolated peptide comprising a sequence of amino acid residues comprising WLPFGFILV (SEQ ID NO:2).
9. An isolated peptide comprising a sequence of amino acid residues comprising ALPFGFILV (SEQ ID NO:3).
10. An isolated peptide comprising a sequence of amino acid residues comprising ELPFGFILV (SEQ ID NO:4).
11. A composition comprising the peptide of any of claims 1 or 8-10 and a physiologically-acceptable carrier.
12. The composition of claim 11, wherein said carrier is a pharmaceutically-acceptable carrier.

13. The composition of claim 11, further comprising a T-helper epitope.
14. The composition of claim 13, wherein the T-helper epitope is a peptide comprising a sequence of amino acids consisting essentially of TPPAYRPPNAPIL (SEQ ID NO:5).
15. The use of the peptide of any of claims 1 or 8-10 to prepare a medicament useful as a vaccine against glioma.
16. The use of the composition of claim 11 to prepare a medicament useful as a vaccine against glioma.
17. A method of vaccinating a patient against glioma, comprising introducing into the patient a peptide of any of claims 1 or 8-10 under conditions sufficient for said patient to develop a cytotoxic T-cell lymphocyte (CTL) response.
18. A method of vaccinating a patient against glioma, comprising introducing into the patient a composition according to claim 11 under conditions sufficient for said patient to develop a cytotoxic T-cell lymphocyte (CTL) response.
19. The method of claim 17, wherein said patient is a mammal.
20. The method of claim 18, wherein said patient is a mammal.
21. The method of claim 19, wherein said patient is human.
22. The method of claim 20, wherein said patient is human.
23. The use of a peptide having the sequence TLADFDPRFV (SEQ ID NO:6) to prepare a medicament useful as a vaccine against glioma.
24. A method of vaccinating a patient against glioma, comprising introducing into the patient a peptide having the sequence TLADFDPRFV (SEQ ID NO:6) under conditions sufficient for said patient to develop a cytotoxic T-cell lymphocyte (CTL) response.

25. The use of a composition comprising a peptide having the sequence TLADFDPRFV (SEQ ID NO:6) and a physiologically acceptable carrier to prepare a medicament useful as a vaccine against glioma.
26. A method of vaccinating a patient against glioma, comprising introducing into the patient a composition comprising a peptide having the sequence TLADFDPRFV (SEQ ID NO:6) and a physiologically acceptable carrier under conditions sufficient for said patient to develop a cytotoxic T-cell lymphocyte (CTL) response.
27. The use of claim 25 wherein said physiologically acceptable carrier is a pharmaceutically-acceptable carrier.
28. The method of claim 26 wherein said physiologically acceptable carrier is a pharmaceutically-acceptable carrier.
29. The use of claim 25, wherein said composition further comprises a T-helper epitope.
30. The method of claim 26, wherein said composition further comprises a T-helper epitope.
31. The use of claim 29, wherein the T-helper epitope is a peptide comprising a sequence of amino acids consisting essentially of TPPAYRPPNAPIL (SEQ ID NO:5).
32. The method of claim 30, wherein the T-helper epitope is a peptide comprising a sequence of amino acids consisting essentially of TPPAYRPPNAPIL (SEQ ID NO:5).
33. The use of claim 23 or 25, wherein the glioma is ependymoma, astrocytoma, oligodendrogloma, glioblastoma, or a mixed glioma.
34. The method of claim 24 or 26, wherein the glioma is ependymoma, astrocytoma, oligodendrogloma, glioblastoma, or a mixed glioma.
35. The method of claim 24 or 26, wherein said patient is a mammal.
36. The method of claim 24 or 26, wherein said patient is human.

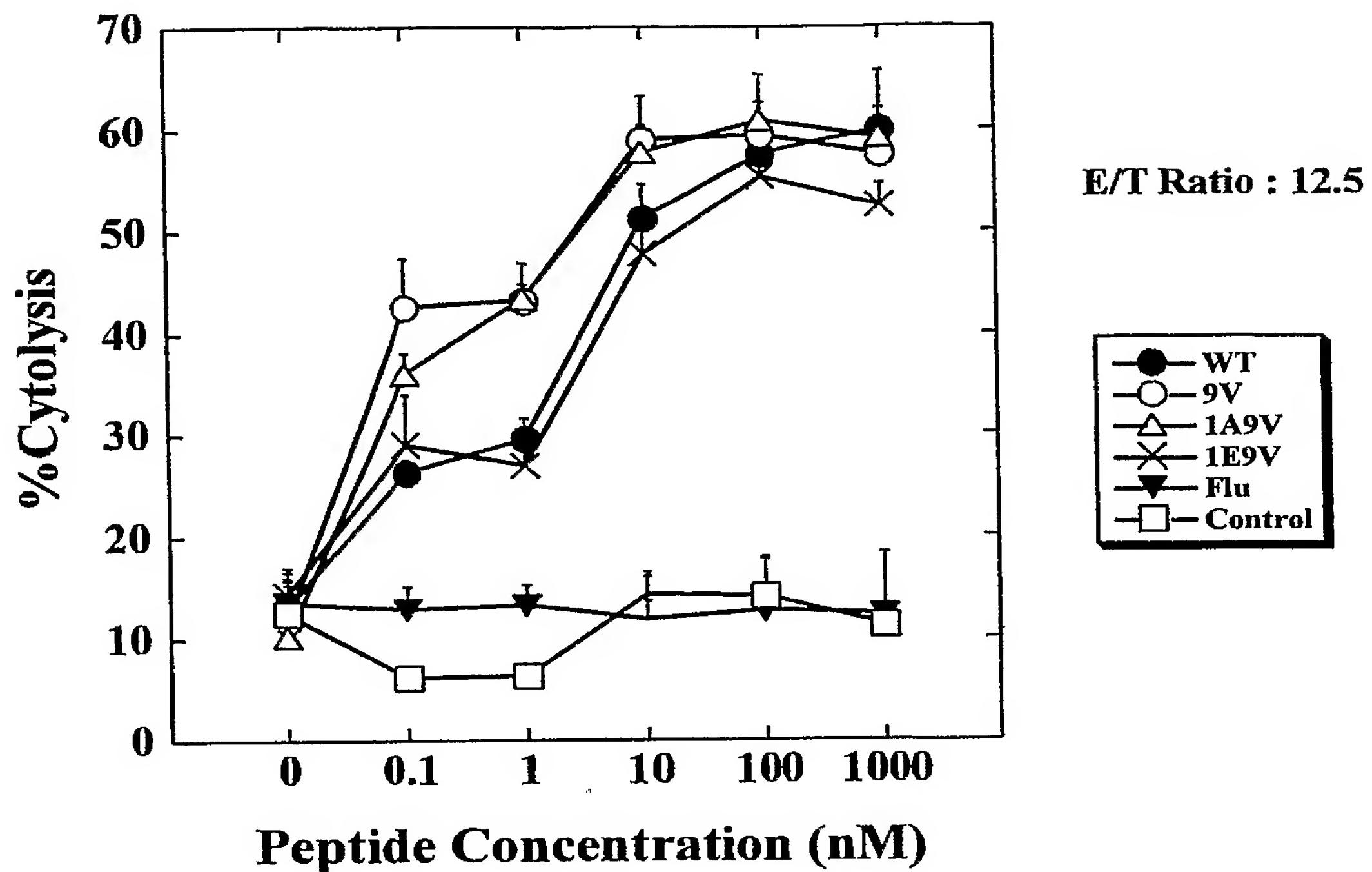
Figure 1

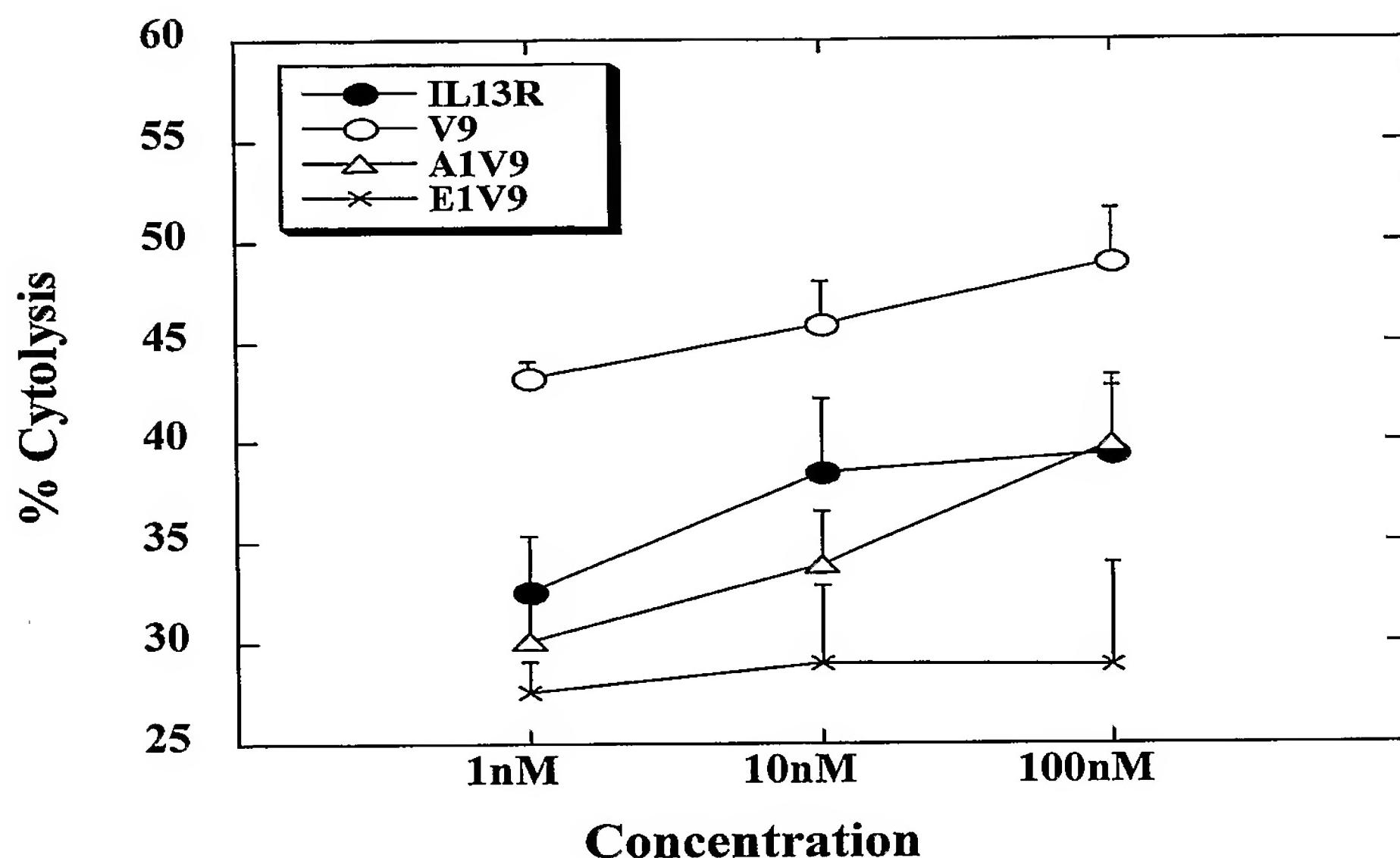
Figure 2

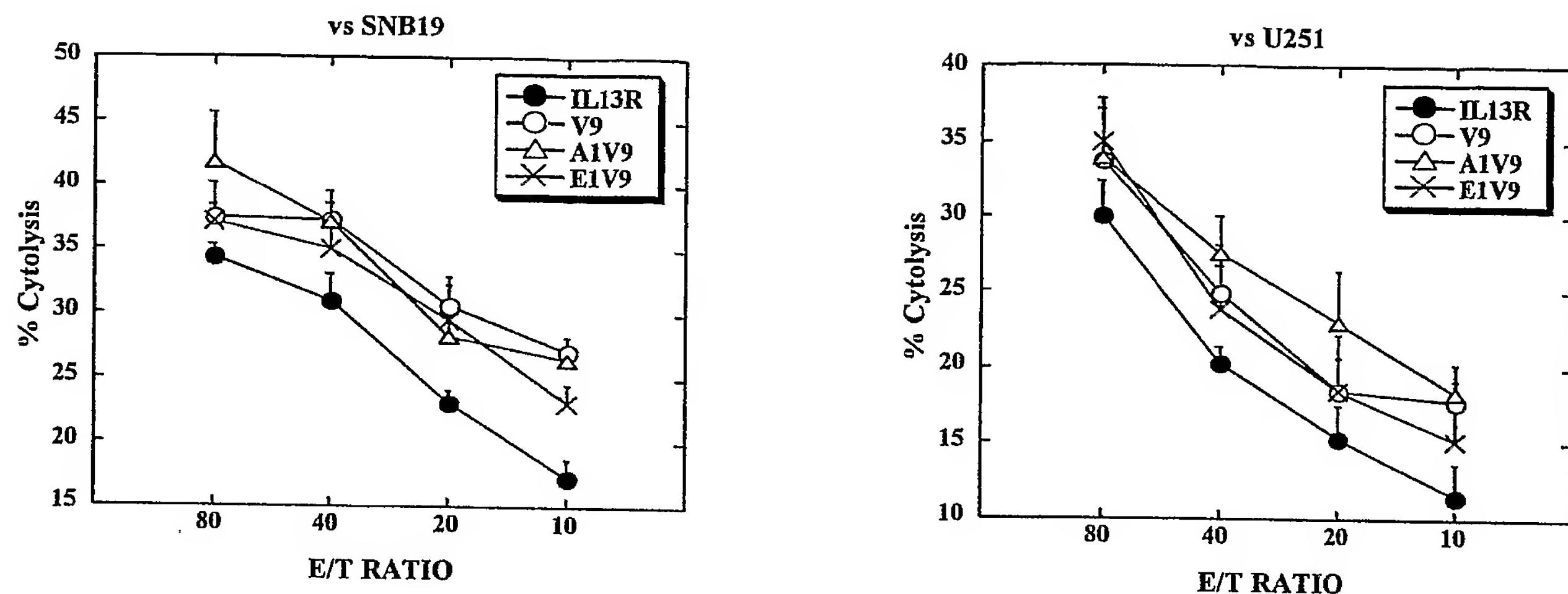
Figure 3

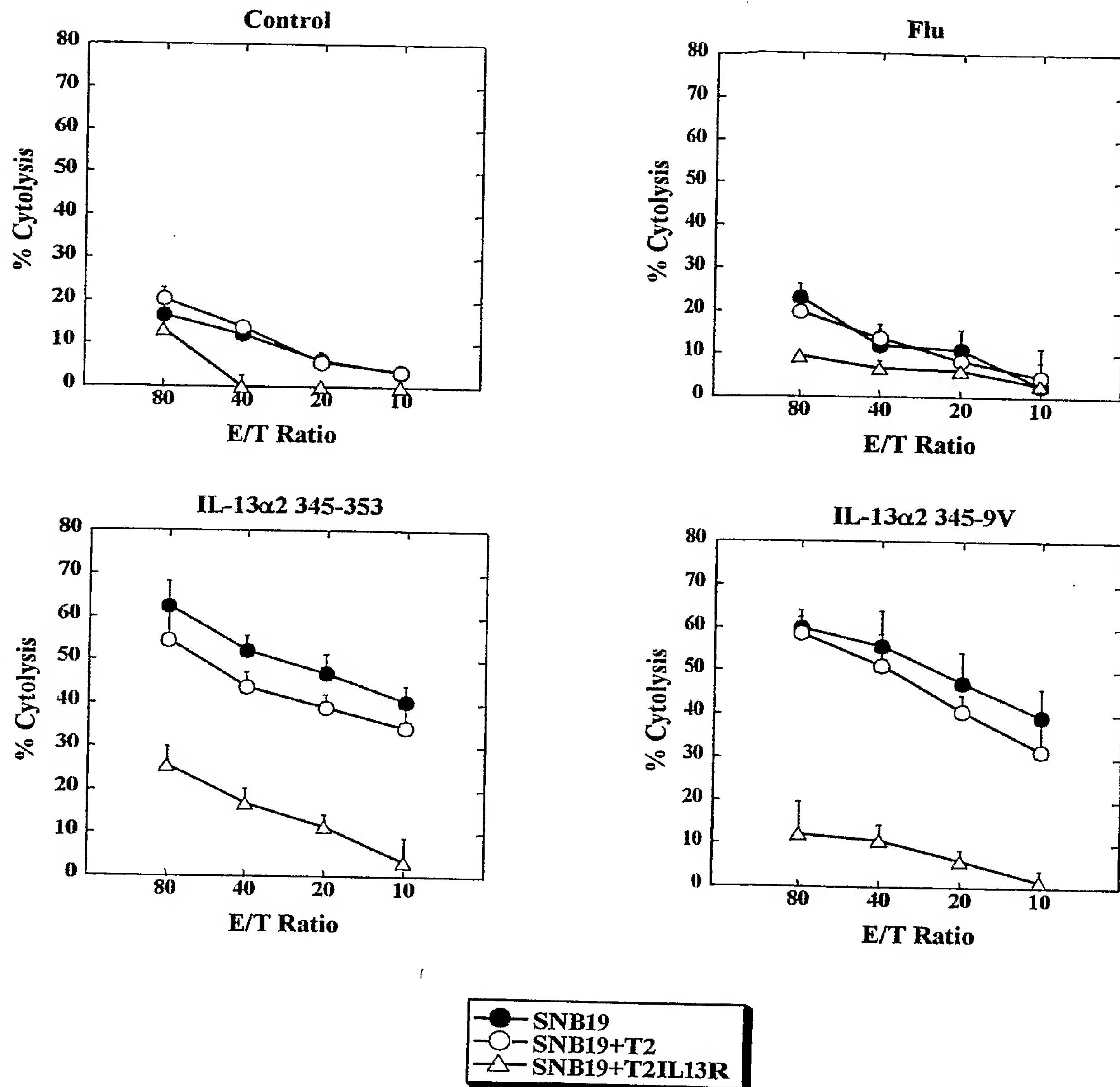
Figure 4

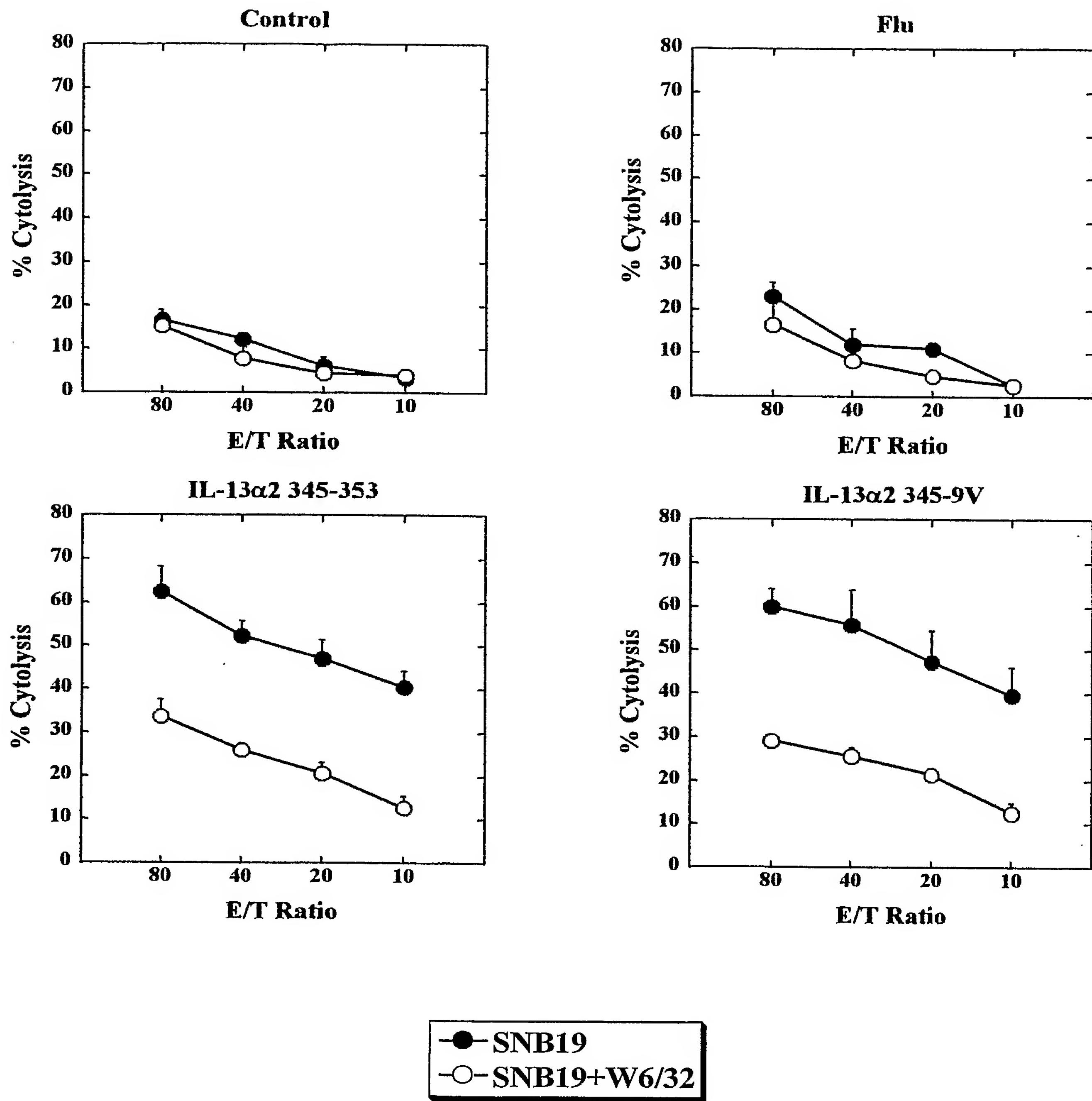
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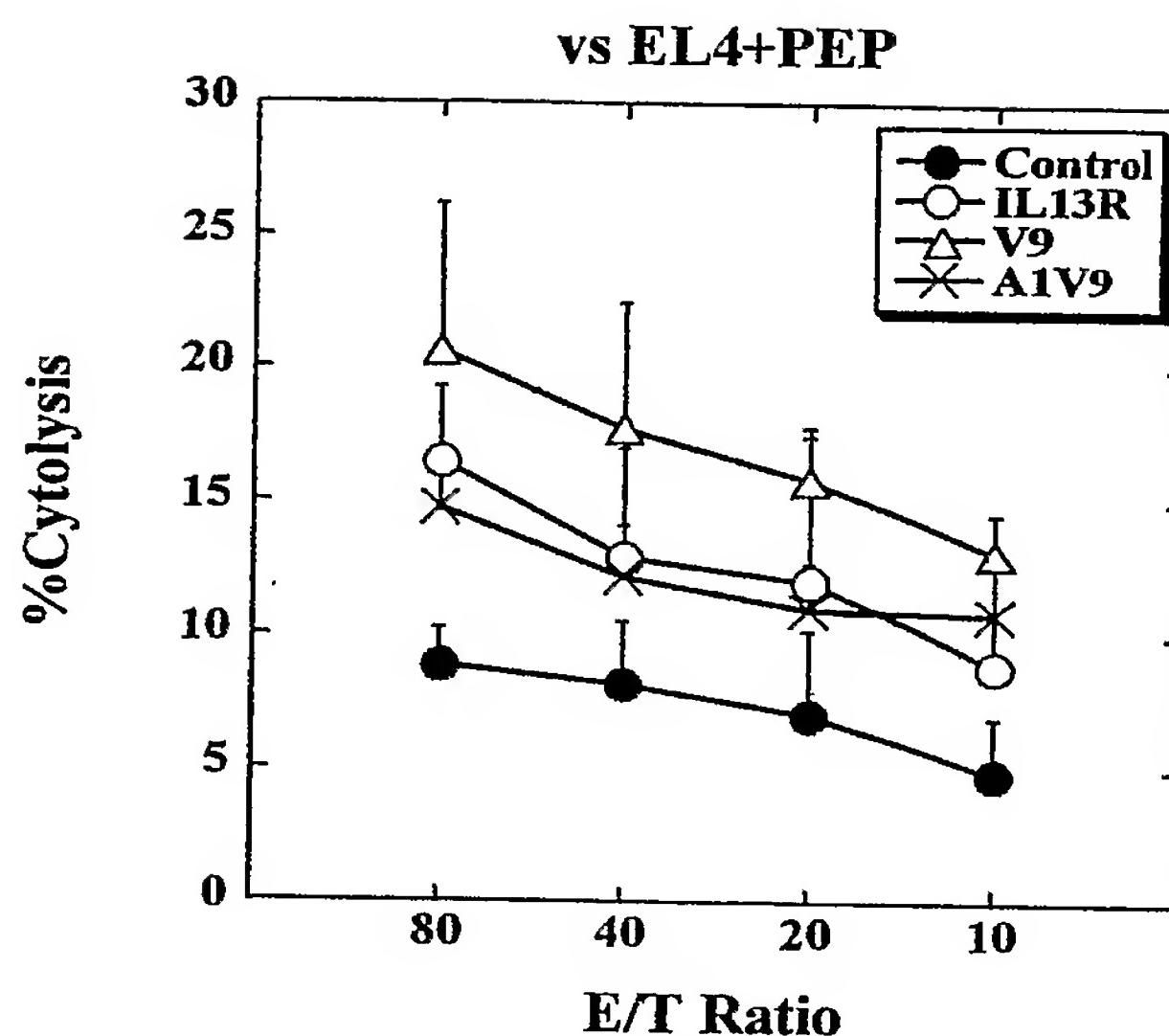
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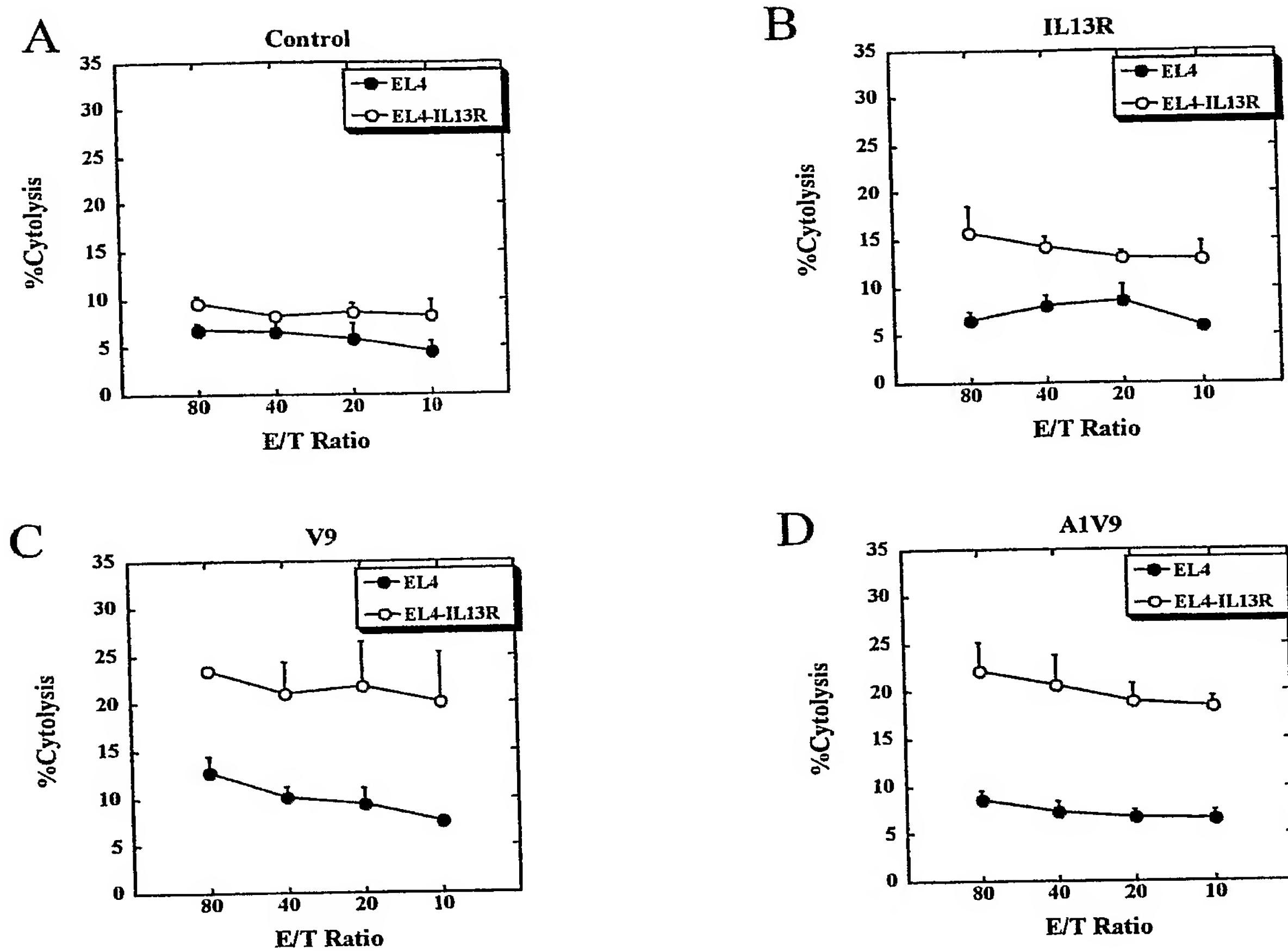
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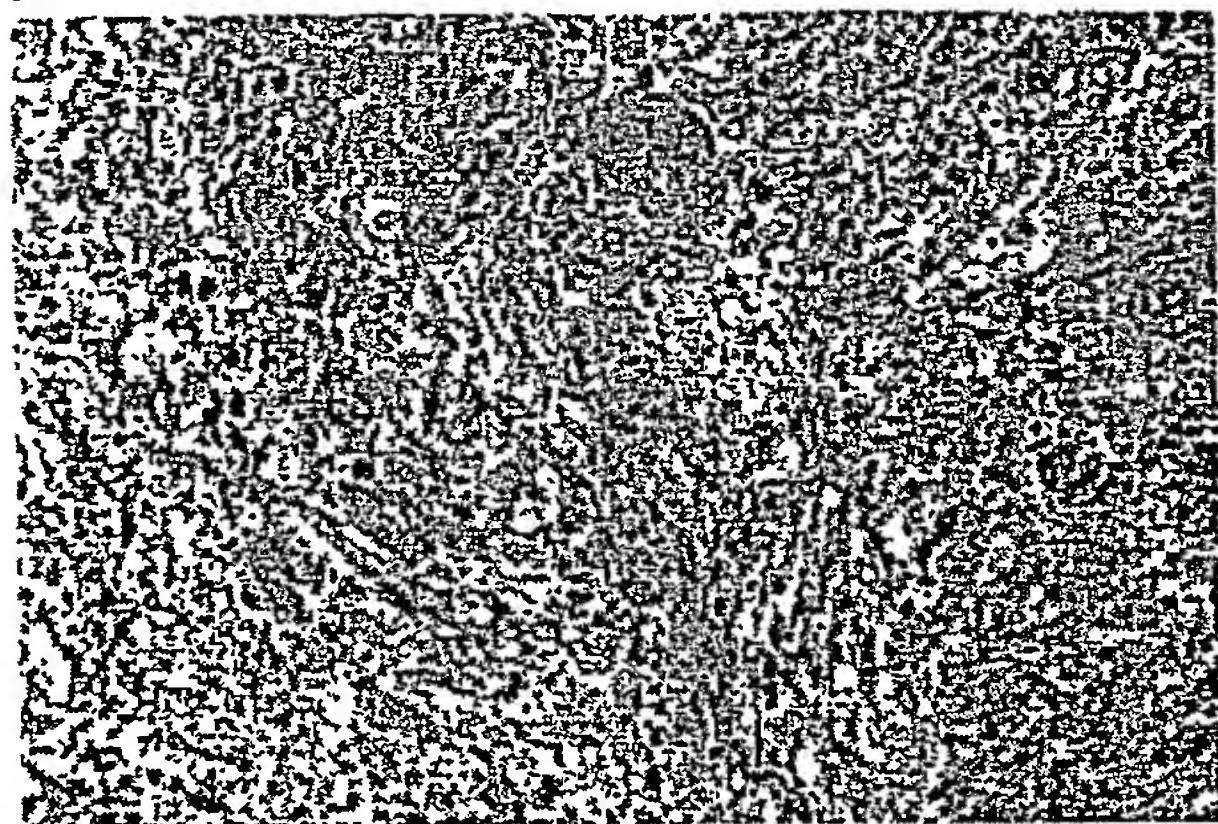
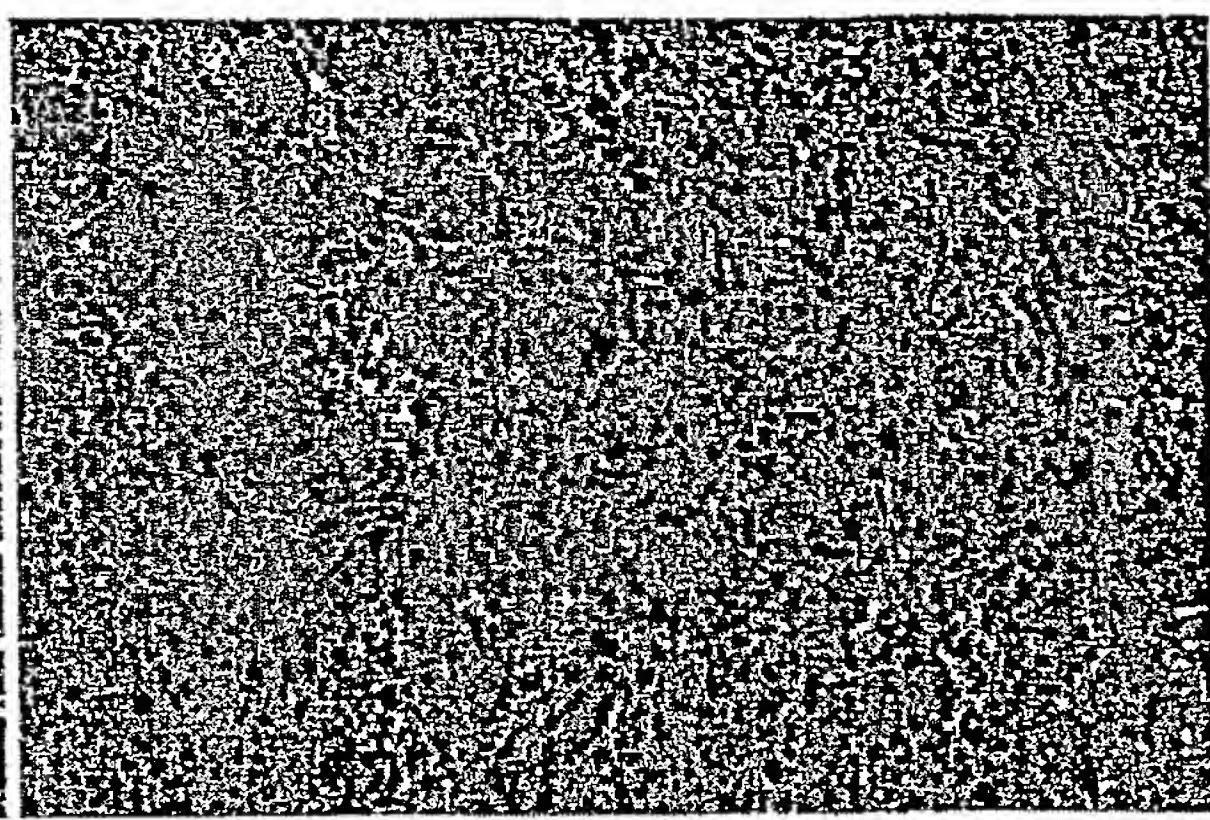
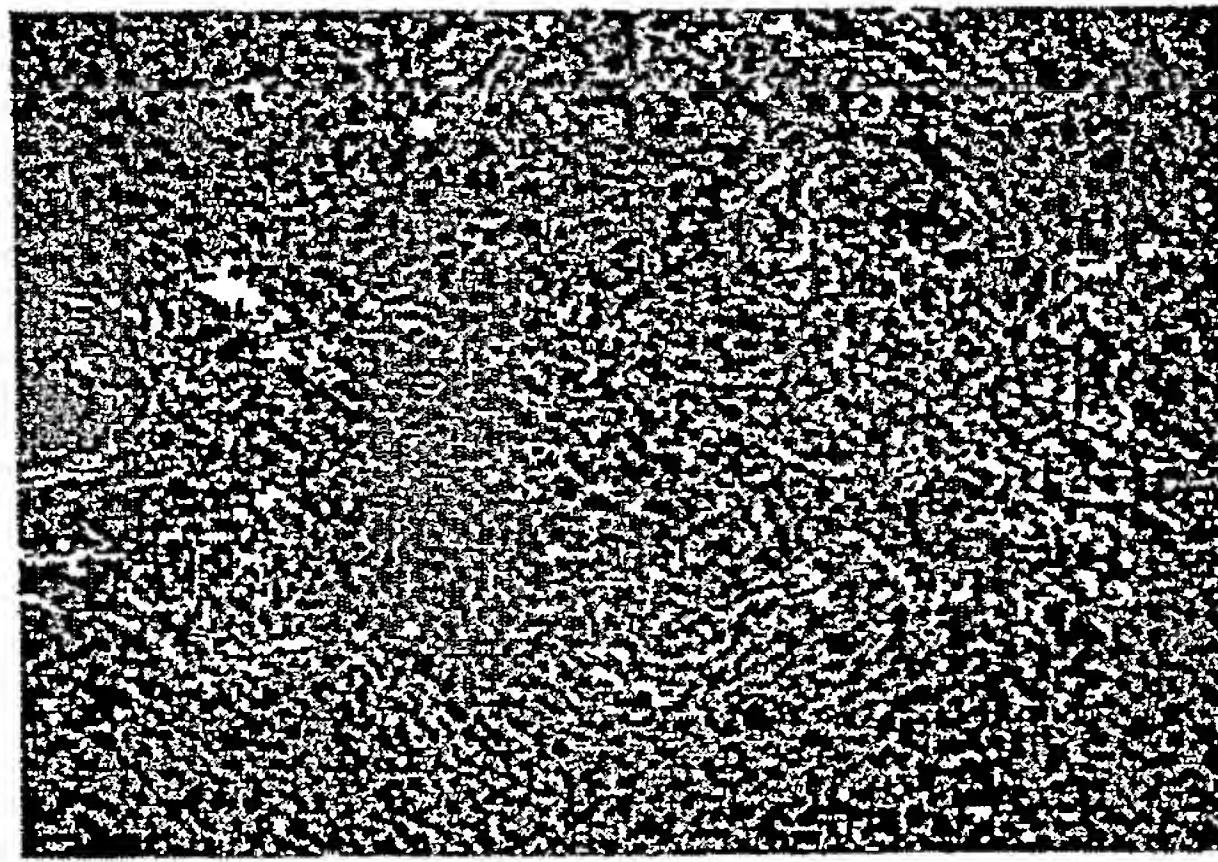
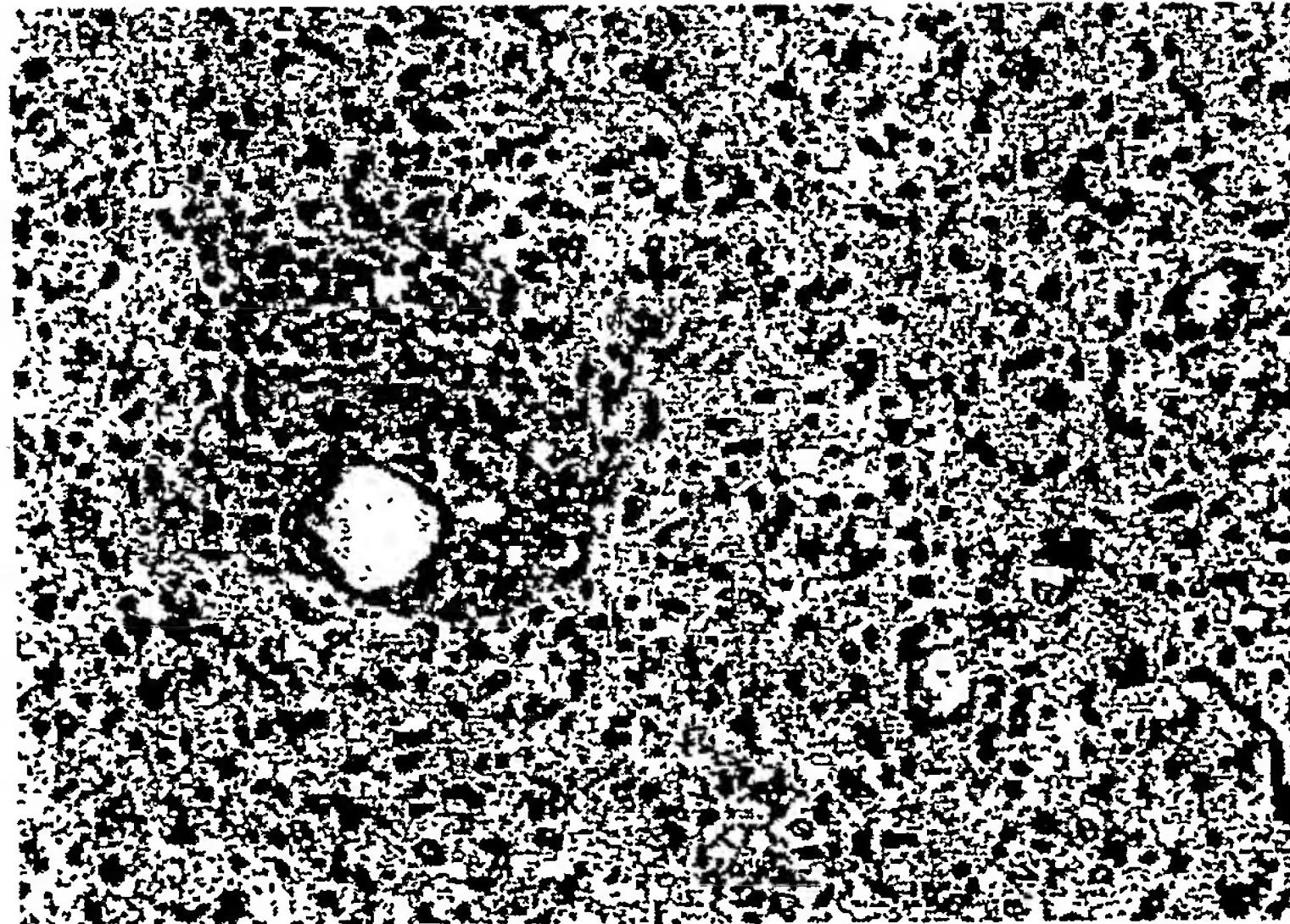
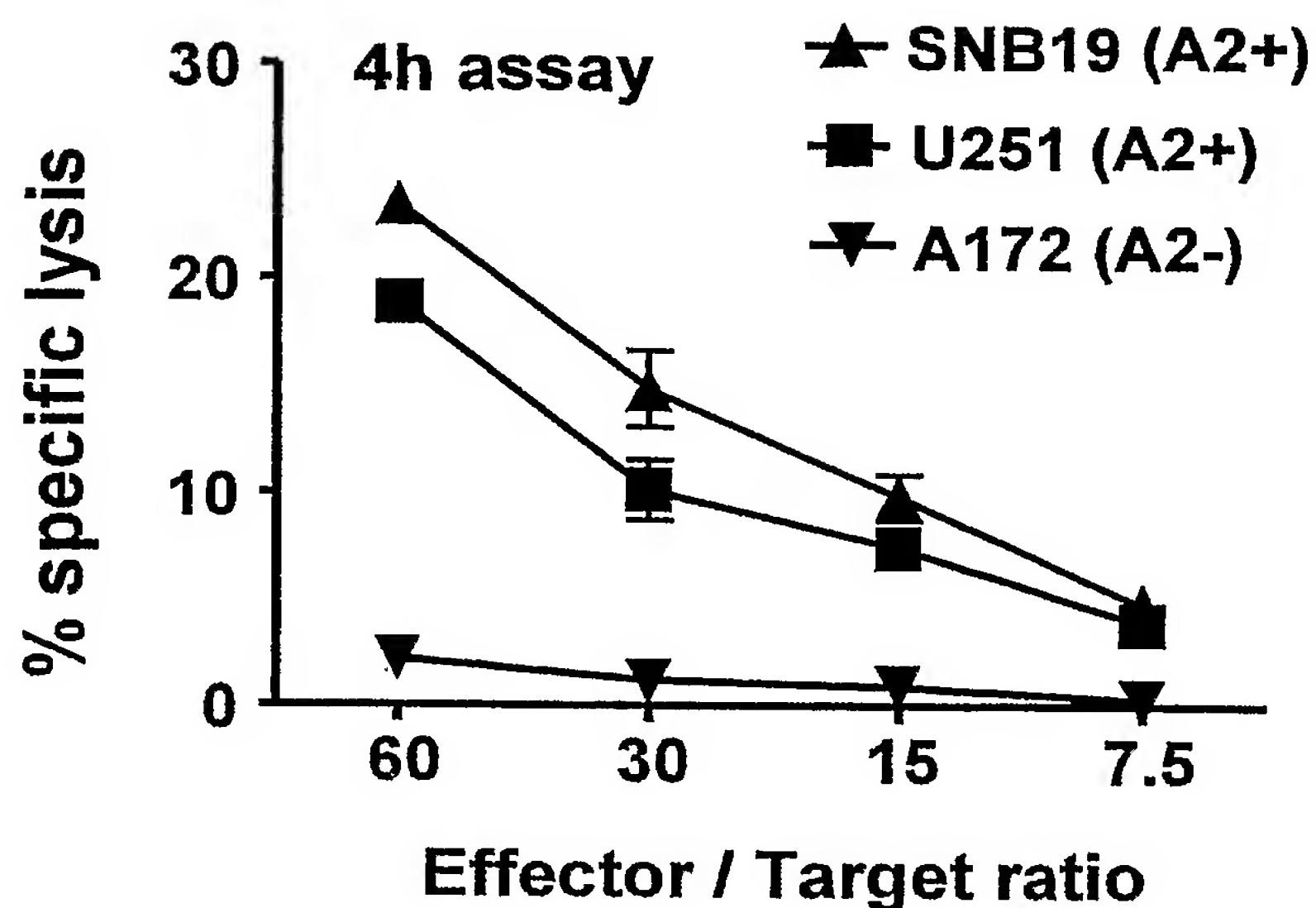
Figure 8**A****B****C****D**

Figure 9

SEQUENCE LISTING

<110> University of Pittsburgh of the Commonwealth System of
Higher Education
Storkus, Walter
Sato, Hidemitsu
Okada, Hideho
Eguchi, Junichi

<120> PEPTIDE ANALOGS CAPABLE OF ENHANCING STIMULATION OF A
GLIOMA-SPECIFIC CTL RESPONSE

<130> 238676

<150> US 60/611,797

<151> 2004-09-21

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(21) International Application Number:
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(54) Title: PEPTIDE ANALOGS CAPABLE OF ENHANCING STIMULATION OF A GLIOMA-SPECIFIC CTL RESPONSE

(57) Abstract: The invention provides a peptide derived from the interleukin-13 receptor $\alpha 2$, which serves as a HLA-A2-restricted cytotoxic T lymphocyte (CTL) epitope. The invention can be used as a vaccine for glioma and can be formulated into compositions for medical or veterinary use. In addition, the invention provides the use of a peptide derived from the Eph family of tyrosine kinase receptors which can be also used as a vaccine for glioma and can be formulated into compositions for medical or veterinary use.

WO 2006/034334 A3

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2005/033794

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C07K14/715 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OKANO FUMIYOSHI ET AL: "Identification of a novel HLA-A*0201-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor alpha2 chain" CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 8, no. 9, September 2002 (2002-09), pages 2851-2855, XP002300523 ISSN: 1078-0432 cited in the application the whole document -----	1-7, 11-22
X	WO 01/58479 A (THE PENN STATE RESEARCH FOUNDATION) 16 August 2001 (2001-08-16) The whole document; see particularly page 13, line 16 to page 14, line 23; example 3 ----- -/-	1-7, 11-22



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"&" document member of the same patent family

Date of the actual completion of the international search

23 March 2006

Date of mailing of the international search report

29.06.2006

Name and mailing address of the ISA/

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Authorized officer

Groenendijk, M

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/033794

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95/22317 A (CYTEL CORPORATION) 24 August 1995 (1995-08-24) example 4 -----	13,14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/033794

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 17-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-22

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 17-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

Claims Nos.:

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-22

Compounds defined in claims 1,8-10, their compositions and use

2. claims: 23-36

Use of the compound defined in claim 23 and its compositions

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2005/033794

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 0158479	A	16-08-2001	AU CA EP JP	3689201 A 2398136 A1 1257289 A1 2004511425 T		20-08-2001 16-08-2001 20-11-2002 15-04-2004
WO 9522317	A	24-08-1995	AT AU CA DE DE EP	277633 T 1847395 A 2183416 A1 69533594 D1 69533594 T2 0804158 A1		15-10-2004 04-09-1995 24-08-1995 04-11-2004 16-02-2006 05-11-1997